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Variable Effects of Exposure to Formulated Microbicides on Antibiotic Susceptibility in Firmicutes and Proteobacteria

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Key words Microbicides, biocides, antibiotics, susceptibility, resistance, formulation.

Running title: Antibiotic susceptibility following exposure to microbicides

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33 ABSTRACT

34 Microbicides are broad-spectrum antimicrobial agents that generally interact with multiple
35 pharmacological targets. Whilst they are widely deployed in disinfectant, antiseptic and
36 preservative formulations, data relating to their potential to select for microbicide or antibiotic
37 resistance have been generated mainly by testing the compounds in much simpler aqueous
38 solutions. In the current investigation, antibiotic susceptibility was determined for bacteria
39 that had previously exhibited decreased microbicide susceptibility following repeated
40 exposure to microbicides either in formulation with sequestrants and surfactants or in simple
41 aqueous solution. Statistically significant increases in antibiotic susceptibility occurred
42 for 12% of bacteria after exposure to microbicides in formulation *vs* 20% after exposure to
43 aqueous solutions, whilst 22% became significantly less susceptible to the antibiotics,
44 regardless of formulation. Of the combinations of bacterium and antibiotic for which British
45 Society for Antimicrobial Chemotherapy breakpoints are available, none became resistant.
46 Linear modeling, taking into account phylogeny, microbicide, antibiotic and formulation
47 identified small but significant effects of formulation that varied depending on bacterium and
48 microbicide. Adaptation to formulated benzalkonium chloride in particular was more likely to
49 increase antibiotic susceptibility than the simple aqueous solution. In conclusion, bacterial
50 adaptation through repeated microbicide-exposure was associated with both increases and
51 decreases in antibiotic susceptibility. Formulation of the microbicide to which the bacteria had
52 previously adapted had an identifiable effect on antibiotic susceptibility but this was typically
53 small relative to the differences observed among microbicides. Susceptibility changes
54 resulting in resistance were not observed.

55

56 INTRODUCTION

57 Microbicides are broad-spectrum antimicrobial compounds that are widely deployed to
58 control the growth of microorganisms or eliminate them. Applications include the control of
59 biofouling and microbial contamination in industry (1) as well as clinical antisepsis (2-4).
60 They are also used extensively in the domestic environment as hygiene adjuncts and
61 preservatives in a range of formulations including oral care products (5), hand sanitizers (6)
62 and hard surface cleaners (7).

63 The safety of certain microbicide applications has been questioned due to the possibility that
64 long-term microbicide exposure could select for reduced antimicrobial susceptibility in
65 bacteria (8-10). Reduced microbicide susceptibility has been reported for some combinations
66 of bacterium and microbicide (11) and changes in bacterial susceptibility to chemically

67 unrelated antimicrobials such as antibiotics or other microbicides have been reported
68 following laboratory microbicide exposure (12, 13). The mechanisms involved in such cross-
69 resistance include selection for mutations in shared cellular target sites, upregulation of efflux
70 pumps (14), reductions in cell permeability (15) and in some cases, sporulation (16).

71 Evidence that microbicides can select for reduced microbicide susceptibility in the
72 environment is limited, with the majority of reports relating to *in vitro* exposure (17).
73 Similarly, little evidence has emerged to firmly link microbicide/antibiotic cross-resistance to
74 microbicide use (18-21). The majority of studies aiming to better understand the potential
75 risks of resistance through microbicide exposure have exposed bacteria to microbicides in
76 aqueous solution with or without the addition of co-solvents such as dimethyl sulfoxide (22)
77 or ethanol (23). In real use however, microbicides are deployed in products formulated with
78 surfactants, sequestrants and other compounds that can interact with cellular targets to
79 influence antimicrobial potency. As previously reported, such formulation can decrease the
80 frequency and extent of the acquisition of reduced microbicide susceptibility in bacteria (24).
81 Accordingly, evaluating the effects of bacterial exposure to microbicides within a formulation
82 chassis containing surfactants and sequestrants may generate more realistic data on which to
83 base risk assessments on the induction of changes in bacterial susceptibility. In the current
84 investigation we have therefore assessed changes in antibiotic susceptibility in bacteria which
85 have previously exhibited decreases in microbicide susceptibility following repeated exposure
86 to a range of microbicides in simple aqueous solutions and in formulations containing
87 commonly used non-ionic surfactants and sequestrants (24). The microbicides tested reflect
88 those frequently used in consumer products such as laundry detergents, hard surface
89 disinfectants and personal care products. The antibiotics were selected on the basis of their
90 common therapeutic use and their inclusion in a US investigation of links between domestic
91 microbicide use and antibiotic resistance (25).

92 MATERIALS AND METHODS

93 **Bacteria.** *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538,
94 and *Escherichia coli* ATCC 25922 were obtained from Oxoid (Basingstoke, United
95 Kingdom). *Acinetobacter baumannii* MBRG15.1, *Pseudomonas putida* MBRG15.2,
96 *Escherichia coli* MBRG15.4 and *Cronobacter sakazakii* MBRG15.5, were isolated from a
97 domestic kitchen drain biofilm. *Enterococcus faecalis* MRBG15.6 is a wound isolate provided
98 by Angela Oates, The University of Manchester.

99 **Chemicals reagents and growth media.** Bacteriological growth media were
100 purchased from Oxoid (Basingstoke, United Kingdom). All other chemical reagents were
101 purchased from Sigma-Aldrich (Dorset, United Kingdom) unless otherwise stated. Bacterial
102 growth media were sterilized at 121°C and 15 lb/in² for 15 min prior to use. *Pseudomonas*
103 *aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis* were
104 cultured on Tryptone Soy agar and broth. *Acinetobacter baumannii*, *Pseudomonas putida* and
105 *Cronobacter sakazakii* were grown on Wilkins Chalgren agar and broth containing 2%
106 sucrose. All bacteria were incubated aerobically at 37°C for 18 h unless stated otherwise.

107 **Antimicrobials.** The microbicides benzalkonium chloride (BAC), chlorhexidine
108 digluconate (CHX 20% v/v), thymol and triclosan were purchased from Sigma-Aldrich
109 (Dorset, UK). Didecyldimethyl ammonium chloride (DDAC 50% v/v) was purchased from
110 Merck Millipore (Durham, UK). Vantocil (a 20% v/v aqueous solution of polyhexamethylene
111 biguanide (PHMB) was obtained from Arch Chemicals Inc. (Manchester, UK). Glydant (1,3-
112 Dimethylol-5,5-dimethylhydantoin; DMDM hydantoin at 54% v/v) was obtained from Lonza
113 (Bishops Stortford, UK) whilst benzisothiazolinone (BIT) was supplied by Unilever (Port
114 Sunlight, UK). All microbicides were prepared in aqueous solution or added to a microbicide-
115 free formulation chassis containing sequestrants and surfactants as previously described (24),
116 at concentrations reflective of their normal deployment in consumer products. BAC, CHX,

DDAC, DMDM hydantoin, PHMB and thymol were prepared at 1% (v/v) in a general purpose cleaner. Triclosan was added to a laundry detergent at 0.0066% (w/v). Benzisothiazolinone was formulated into a laundry detergent at 0.02% (v/v). Ciprofloxacin (1µg), cephalothin (20µg), ampicillin (10µg), kanamycin (5µg) and tetracycline (10µg) antibiotic discs were obtained from Oxoid (Basingstoke, UK).

16S rRNA gene sequencing. Single bacterial colonies were dispersed in 100µl of nanopure water, vortexed for 30 sec. and boiled at 100°C for 15min. to lyse cells. Microcentrifuge tubes were centrifuged at 16, 000 x g for 1 min to remove cellular debris and the resulting supernatant was retained as DNA template. PCR was performed using the primers 8FLP (5'-GAG TTT GAT CCT GGS TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') at 5µM per reaction. PCR was conducted using a Biometra TGradient thermocycler (Analytik Jena, Germany) and run for 35 thermal cycles: 94°C (1 min), 53°C (1 min) and 72°C (1min). A 15 min. elongation step was included in the final cycle. PCR products were purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) according to manufacturer's instructions and the resulting DNA yield was quantified using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). A reaction mixture containing 4pM forward or reverse primer and 40-50ng of DNA in 10µl total volume was used for DNA sequencing. DNA sequencing was performed using the Applied Biosystems 3730 DNA Analyzer (ThermoFisher, Paisley, UK).

Microbicide exposure in aqueous solution and formulation. A system previously validated as highly selective for changes in antimicrobial susceptibility (26, 27) was used. Reproducible *c.* 100-fold-concentration gradients of the antimicrobial compounds were generated on Tryptone Soy or Wilkins Chalgren agar plates using an automated spiral plater (Don Whitley Scientific, Shipley, United Kingdom). Antimicrobials in aqueous solution or in formulation (50µl) were deposited on the agar surface. Plates were dried for 1h at room

temperature prior to radial deposition of bacterial pure cultures and then incubated (4d; 37°C) in an aerobic incubator. After incubation, growth observed at the highest microbicide concentration was aseptically removed and streaked onto a fresh plate containing the same antimicrobial compound concentration gradient. Where growth was observed across the whole antimicrobial gradient, a new plate produced with a 5-fold-higher microbicide concentration was used. This process was repeated until 14 passages had occurred (P14). Bacteria at P0 and P14 were archived for subsequent susceptibility testing.

Determination of antibiotic susceptibility. Bacteria showing ≥ 4 -fold increases in minimum bactericidal concentration (MBC) after microbicide/formulation exposure were investigated for changes in antibiotic susceptibility. Antibiotic susceptibilities were determined for ciprofloxacin (1 μ g), cephalothin (20 μ g), ampicillin (10 μ g), kanamycin (5 μ g) and tetracycline (10 μ g). Disc diffusion assays were performed according to the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial susceptibility testing (28).

Statistical analyses. Antibiotic zone of inhibition sizes were compared before and after adaptation to microbicides using Mann-Whitney U tests and in the cross-resistance assays using linear mixed effect models (LMMs). LMMs were required to simultaneously compare and account for the effects on the inhibition zone of: a) microbicidal environment to which the bacterium was adapted, b) the antibiotic against which it was tested and c) the interaction of microbicidal environment and antibiotic (each fitted as fixed effects) plus d) the different bacteria (fitted as a random effect), allowing the variation among bacteria to differ for different antibiotics. Initial models with this structure violated the statistical assumptions of normality of residuals and homogeneity of variance. Box-Cox transformation indicated that a transformation with a power of 0.5 (square root) was approximately optimal to address the non-normality and was therefore used. A wide range of different models accounting for non-

homogeneity of variance in response to different variables was tested. Models allowing different variances for different bacteria and different variances for different microbicidal environments were superior to all others tested (lowest Akaike information criterion). To account for the fact that closely related bacteria are likely to respond more similarly than others just through having a more recent common ancestor, a correlation term was included based on the 16S-based phylogenetic tree of the strains used. Testing different weightings on this correlation term (Pagel's λ (29)) determined that a Brownian model (i.e. Pagel's $\lambda = 1$) was best. In addition, a LMM was fitted for the subset of data involving microbicides where bacteria were tested that had adapted to both formulated and unformulated versions of the microbicidal environment. In this case, accounting for non-homogenous variance was best done by allowing different variances for different microbicidal environments and for variance to increase at higher values according to the formula $e^{(0.65 * \text{zone of clearance value})}$. All models were fitted using the NLME package (Version 3.1) (30) in R version 3.2 (31) with phylogenetic correlation structures created using the APE package (version 3.3) (32). Where p-values are not explicitly given, statistical significance was deemed to be $p < 0.05$.

RESULTS

After exposure to microbicides in simple aqueous solution, out of 90 possible combinations of bacterium and antibiotic, 22% significantly ($P < 0.05$) reduced in antibiotic susceptibility (8% towards ciprofloxacin, 6% to ampicillin, 4% to kanamycin, 2% to tetracycline and 2% to cephalothin). In comparison, 20% significantly increased in antibiotic susceptibility (6% towards ciprofloxacin, 4% to kanamycin, 4% to tetracycline, 3% to cephalothin and 2% to ampicillin). After exposure to the formulated microbicides, out of 50 possible combinations of bacterium and antibiotic, 22% significantly decreased in antibiotic susceptibility (6% ciprofloxacin, 6% kanamycin, 4% cephalothin and 4% tetracycline and 2% ampicillin). In comparison, 12% significantly increased in antibiotic susceptibility (8% ciprofloxacin 2%

kanamycin and 2% tetracycline). Importantly, whilst statistically significant increases and decreases in antibiotic susceptibility occurred, generation of resistance as defined by BSAC breakpoints was not observed in any previously susceptible bacterium.

The frequency of reduction in antibiotic susceptibility was highest in organisms exhibiting previously reduced susceptibility towards DMDM hydantoin (80%), followed by BAC, CHX, DDAC (20%), triclosan (20%) and PHMB (16%). Bacteria with reduced susceptibility to triclosan showed the highest frequency of increased antibiotic susceptibility (45%), followed by CHX (30%), DDAC (27%), DMDM hydantoin (20%) and PHMB (4%). In comparison, after exposure to the formulations, 27% of thymol formulation and 20% of DDAC formulation-adapted isolates exhibited increased antibiotic susceptibility, whilst 40% of DDAC formulation, 33% of thymol formulation, 10% of BAC formulation and 7% of PHMB formulation-adapted bacteria had significantly decreased antibiotic susceptibility. The following section details the effects of each microbicide on antibiotic susceptibility.

Benzalkonium chloride. When comparing unexposed to BAC-adapted organisms there was a significant decrease in susceptibility of *S. aureus* to ciprofloxacin and kanamycin (Table 1). *E. coli* also showed a significant reduction in kanamycin susceptibility after exposure to BAC. After repeated exposure to BAC formulation *S. aureus* showed a significantly decreased susceptibility to ciprofloxacin (Table 1).

Chlorhexidine. *S. aureus* showed a significant decrease in susceptibility to ampicillin and ciprofloxacin after CHX exposure as well as an increase in susceptibility to tetracycline (Table 1). *E. coli* demonstrated increased susceptibility to ciprofloxacin and ampicillin after repeated exposure to chlorhexidine.

Didecylmethyl ammonium chloride. After exposure to DDAC, *A. baumannii* showed a significant increase in susceptibility to ciprofloxacin and kanamycin and decreased susceptibility to tetracycline when compared to the bacterium before microbicide exposure

(Table 1). Increased susceptibility to ciprofloxacin, kanamycin and cephalothin was observed for the *E. coli* drain isolate, whilst a significant reduction in tetracycline susceptibility was also evident in this bacterium. After exposure to DDAC in formulation, the *E. coli* drain isolate underwent a significant reduction in kanamycin, cephalothin, tetracycline and ampicillin susceptibility, and an increase in susceptibility to ciprofloxacin. *P. aeruginosa* showed a significant increase in ciprofloxacin susceptibility after long-term exposure to DDAC formulation (Table 1).

DMDM hydantoin. After repeated exposure to DMDM hydantoin the *E. coli* drain isolate demonstrated a significant reduction in ciprofloxacin, kanamycin, cephalothin and ampicillin susceptibility and an increase in tetracycline susceptibility when compared to its pre-exposed counterpart (Table 1).

Polyhexamethylene biguanide. Following adaptation to PHMB, the *E. coli* drain isolate exhibited a decrease in kanamycin and ciprofloxacin susceptibility (Table 1). *S. aureus* developed a significantly reduced susceptibility to ampicillin and ciprofloxacin after repeated PHMB exposure but higher tetracycline susceptibility when compared to the unexposed parent strain. After exposure to PHMB formulation *S. aureus* also showed a significant reduction in ciprofloxacin susceptibility.

Thymol. None of the test bacteria demonstrated a significant change in antibiotic susceptibility after exposure to thymol in aqueous solution. Following exposure to the thymol-containing formulation however, *P. putida* underwent significant decreases in susceptibility to ciprofloxacin and kanamycin (Table 1), whilst *E. coli* showed significant increases in ciprofloxacin and cephalothin susceptibility but decreases in susceptibility to kanamycin and tetracycline. *A. baumannii* increased in susceptibility to ciprofloxacin, kanamycin and tetracycline compared to its unexposed counterpart (Table 1).

Triclosan. Following exposure to triclosan, *S. aureus* exhibited significant reductions in ciprofloxacin and ampicillin susceptibility whilst susceptibility to kanamycin, tetracycline and cephalothin increased (Table 1). *E. coli* showed increased susceptibility to ampicillin and ciprofloxacin for this bacterium after triclosan exposure, whilst the *E. coli* drain isolate showed decreased ciprofloxacin susceptibility but increased cephalothin susceptibility, when compared to the parent strain. Comparatively *C. sakazakii* showed a significant increase in ciprofloxacin, cephalothin and kanamycin susceptibility, and a decrease in ampicillin susceptibility after repeated triclosan exposure (Table 1).

To gain an overview of the statistical significance of the observed changes in antibiotic susceptibility and ask whether it was possible to identify consistent patterns in susceptibility, linear mixed-effects models were fitted for how the susceptibility to particular antibiotics varied, dependent on the antibiotic in question, the bacterium and the microbicidal environment previously adapted to. A highly significant interaction ($F_{40, 298} = 15$, $P < 2 \times 10^{-16}$) indicative of different responses to particular antibiotics dependent on the microbicidal environment to which the organism had previously adapted (Fig. 1) was observed. Bacterial strains differed most in their response to ampicillin (standard deviation among strains = 5.1) and least in their response to tetracycline (standard deviation among strains = 2.7), with the responses of different strains to some antibiotics being associated either positively (cephalothin and ampicillin, $r = 0.95$) or negatively (ciprofloxacin and ampicillin, $r = -0.28$), (Table 2).

Data presented in Fig. 1 indicate differences in the antibiotic susceptibility of organisms previously adapted to either formulated or unformulated microbicides. The differences in susceptibility changes observed between microbicides in simple aqueous solution or in complex formulation were highly significant (likelihood ratio test of the full model against a

model treating formulated and unformulated versions of microbicides as equivalent: $LR_{88,70} = 61$, $P = 8.6 \times 10^{-10}$). To test whether there was any consistent effect of formulation; a second linear mixed-effects model was created for the subset of the data where strains had adapted to both formulated and unformulated versions of the same microbicide (PHMB, BAC and DDAC). This indicated that the way bacteria adapted to formulated versus non-formulated versions of a microbicide depended on the microbicide in question ($F_{2, 145} = 4.5$, $P = 0.012$), although that did not vary significantly among the antibiotics ($F_{8, 145} = 0.70$, $P = 0.69$). The effect of formulation was specific to BAC, with formulation giving a small increase in the antibiotic susceptibility of microbes adapted to it (Fig. 2).

DISCUSSION

Investigations into the potential of microbicides to select for reduced microbicide susceptibility in bacteria and induce cross-resistance to antibiotics have been largely conducted by evaluating susceptibility changes following exposure of bacteria to microbicides in simple aqueous solution (17). In such experiments, susceptibility of the exposed bacteria has been reported to decrease for certain combinations of bacterium and microbicide either transiently or stably (26). In the real world however microbicides are deployed in complex formulations containing sequestrants, surfactants and other compounds. Recent investigations indicate that the formulation of microbicides can significantly enhance antibacterial potency and that decreases in microbicide susceptibility after sub-lethal microbicide exposure may be significantly lower in frequency and extent when the microbicides are incorporated into formulations reflecting application in the real world (24, 33). This highlights the value of risk assessments that more accurately reflect the way microbicides are deployed. In the current investigation we have evaluated whether the formulation of microbicides additionally mitigates the development of antibiotic insusceptibility in bacteria.

In order to investigate whether the formulation of microbicides affects cross-resistance to antibiotics, we studied the induction of changes in antibiotic susceptibility in bacteria that had been repeatedly exposed, using a highly selective system arguably representing a worst case scenario, to microbicides in simple aqueous solution and in formulation with ingredients that are used in consumer products such as laundry detergents, hard surface disinfectants and personal care products (24). It should be noted that whilst the majority of microbicides tested are widely used in domestic cleaning products, the use of triclosan in Europe is generally restricted to applications where its utility is greatest, such as oral care.

Out of 288 microbicide-exposed bacteria, 28 organisms previously demonstrated a ≥ 4 -fold decrease in microbicide susceptibility (18 organisms adapted to microbicides following exposure to simple aqueous solutions and 10 to microbicides in formulation). These were further evaluated for changes in antibiotic susceptibility in the current study. The difference in the numbers of test bacteria between treatment groups results from the mitigating effects that the formulation of microbicides had on the development of microbicide insusceptibility. Increases in antibiotic susceptibility occurred at higher frequency following exposure to simple solutions in comparison to formulations (20% v 12%) whilst 22% became significantly less susceptible to the antibiotics regardless of formulation. Whilst both increases and decreases in antibiotic susceptibility were observed in the test bacteria after exposure to microbicide/formulation, no bacterium became resistant according to published BSAC breakpoints.

Changes in antibiotic susceptibility varied between the test antibiotics, bacteria and the microbicides that the bacteria had been previously adapted to, suggesting little correlative effect between the different variables. One positive correlation was however observed between the β -lactam antibiotics ampicillin and cephalothin (Table 2). In this case,

microbicide exposure could have altered alteration transpeptidase expression or otherwise influenced cell wall permeability, subsequently impacting on the efficacy of these antibiotics which target cell wall synthesis.

In some cases, bacterial antibiotic susceptibility was increased following microbicide exposure. It is notable that such “cross-susceptibility” was associated with adaptation to at least some microbicides for all antibiotics except ampicillin (Fig. 1). The phenomenon of “cross-susceptibility” has been observed in several previous investigations (17, 22, 34, 35) where links between antibiotics and decreased microbicide susceptibility in bacteria have been demonstrated *in vitro* (14, 17). In a recent study, exposure of *Burkholderia cepacia* to low concentrations of either CHX or BAC resulted in variable reductions in antibiotic susceptibility (36). CHX exposure was reportedly associated with significant decreases in susceptibility to ceftazidime, ciprofloxacin and imipenem, whilst short-term exposure to BAC resulted in significant decreases in ceftazidime, ciprofloxacin and meropenem susceptibility. These effects were however highly variable between biological replicates in a manner suggestive of stochastic effects. In another recent investigation, six *S. aureus* strains including methicillin-resistant *S. aureus* were repeatedly exposed to triclosan. Susceptibility to triclosan was significantly decreased in all exposed bacteria, whereas antibiotic susceptibility was significantly increased in the majority of cases. Whilst the reasons for cross-susceptibility have not been elucidated, they are likely to include general fitness costs of adaptation and transient cellular damage as previously hypothesized (37).

Mechanisms of cross-resistance have been more extensively investigated and include non-specific reductions in cell permeability, active efflux of the compound from the bacterial cell or acquired mutations in shared target sites (14, 17). Antibiotics such as aminoglycosides enter the cell through a mechanism of self-promoted uptake (38) whereby they displace

cations in the bacterial cell envelope leading to the reorganisation of lipopolysaccharide, which may facilitate antibiotic entry. This mechanism of self-promoted uptake mirrors that of polymeric biguanides, such as PHMB and CHX (39) which has led to the question as to whether any adaptation to reduce biguanide uptake may have a resulting effect on the uptake of aminoglycosides into the bacterial cell. The current investigation included the evaluation of any changes in susceptibility to the aminoglycoside antibiotic kanamycin in bacteria that had previously shown reduced susceptibility to both CHX and PHMB. However, we found no evidence of a systematic effect of this sort (indeed adaptation to CHX typically led to an increase in susceptibility to kanamycin; Fig. 1) and only the PHMB adapted *E. coli* drain isolate showed any significant reduction in antibiotic susceptibility (Table 1).

Cross-resistance between quaternary ammonium compounds (QACs), such as BAC and DDAC and antibiotics has been attributed to the expression of broad-range efflux systems capable of removing both the microbicide as well as certain antibiotics from the bacterial cell (40-42). It has additionally been noted that genes encoding QAC-specific efflux pumps such as *qacA/B* may be detected on plasmids bearing β -lactamases in certain clinical isolates, suggesting another cause for correlation between QACs and penicillins, such as ampicillin (43). Furthermore, the *qacE* gene has been detected in the 3' conserved sequence of certain integrons found in multiple Gram-negative bacteria. Integrons often contain multiple antibiotic resistance genes, and due to their high mobility, may allow the dissemination of both QAC and antibiotic resistance genes through a population via horizontal gene transfer (44). Our data indicate that 20% of bacterial isolates with reduced BAC and DDAC susceptibility in addition to 40% and 10% of isolates with reduced DDAC or BAC formulation susceptibility, were also significantly reduced in their antibiotic susceptibility. Linear mixed effect modelling revealed that the formulation of BAC conferred a moderate protective effect on the development of antibiotic cross-resistance (Fig. 2), possibly

suggesting a regulatory impact of the formulation excipients on the induction of the aforementioned efflux mechanisms, due to non-specific effects on cell permeability or through other cellular changes.

Triclosan exposure may select for mutations in the target enzyme *fabI*, an enoyl-acyl carrier protein reductase that participates in bacterial fatty acid synthesis (45). There has been concern over the induction of cross-resistance between triclosan and therapeutic agents that also share this target enzyme, such as isoniazid used to treat *Mycobacterium tuberculosis*. Cross-resistance between triclosan and certain antibiotics has been reported in *P. aeruginosa* and is largely believed to be due to increased expression of the MexAB-OprM efflux system (14). In the current investigation, data show reductions in ciprofloxacin susceptibility in *S. aureus* and the *E. coli* drain isolate together with reductions in ampicillin susceptibility in *S. aureus* and *C. sakazakii* after repeated triclosan exposure, which may potentially be mediated through regulation of efflux or cell permeability.

Whilst the induction of cross-resistance between microbicides and antibiotics has been previously investigated, little information is available concerning any effect of incorporation of microbicides into formulations containing surfactants and sequestrants on antibiotic susceptibility in adapted bacteria. Data presented here indicate that both decreases and increases in antibiotic susceptibility can occur in bacteria following exposure to microbicides in simple solution and in formulations using a highly selective system. A rigorous statistical analysis demonstrated that formulation significantly affected the development of cross-resistance but that this was variable with the only consistently identified formulation effect being a small increase in susceptibility across antibiotics in strains adapted to the formulated, relative to the unformulated version of the microbicide benzalkonium chloride.

In conclusion, whilst both increases and decreases in antibiotic susceptibility were observed in microbicide and formulation adapted bacteria, these were not sufficient to confer clinical resistance according to published BSAC breakpoints.

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TRANSPARENCY DECLARATION

Alejandro Amézquita is an employee of Unilever. Peter McClure was an employee of Unilever when this project was initiated. All other authors: none to declare.

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Table 1. Antibiotic susceptibility of bacterial isolates that showed a ≥ 4 -fold decrease in microbicide/formulation susceptibility following exposure to microbicides in simple aqueous solution or formulated with surfactants and sequestrants.

Microbicide	Bacterium	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UE	UF	F	UE	UF	F	UE	UF	F	UE	UF	F	UE	UF	F
		P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
BAC	<i>S. aureus</i> [†]	22	14 (0.5)	18 (0.5)	17 (1.5)	14 (0.6)	17 (0.5)	45 (0.5)	43	45	47 (0.5)	45 (0.5)	46	26 (0.5)	25 (0.5)	27 (0.5)
	<i>E. coli</i> [†]	29 (1.5)	31	31 (0.5)	15 (1.2)	12 (0.5)	14 (0.4)	18 (0.5)	16 (2.1)	18	21	22 (0.5)	21	21 (0.5)	21 (0.5)	20 (0.5)
	<i>P. aeruginosa</i> [†]	25 (1.5)	25	na	ns	ns	na	ns	ns	na	ns	ns	na	ns	ns	na
CHX	<i>S. aureus</i> [†]	22	19 (0.5)	na	17 (1.5)	18	na	45 (0.6)	45 (0.5)	na	47 (0.5)	29 (1)	na	26 (0.6)	35 (2.2)	na
	<i>E. coli</i> [†]	29 (1.5)	35 (0.5)	na	15 (1.2)	16 (0.5)	na	18 (0.5)	20 (2.1)	na	21	24 (0.5)	na	21 (0.5)	23 (1.5)	na
DDAC	<i>P. aeruginosa</i> [†]	25 (1.5)	25	28 (0.6)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	<i>A. baumannii</i> [*]	19	27	na	19	21	na	ns	ns	na	ns	ns	na	15	13	na
	<i>E. coli</i> [*]	37	42 (1.5)	40 (0.6)	14	18	11	19	24 (2.1)	15 (0.5)	25	26 (1.5)	21 (0.6)	20	11 (0.5)	11 (0.5)
DMDM	<i>E. coli</i> [*]	37	35	na	14	12 (1.5)	na	19	16	na	25	20 (0.5)	na	20	24	na
PHMB	<i>S. aureus</i> [†]	22	20 (0.5)	21	17 (1.5)	17 (1.2)	16 (0.5)	45 (0.6)	45 (0.5)	45	47 (0.5)	35 (0.5)	45 (1.5)	26 (0.6)	36 (1.5)	25 (0.5)
	<i>E. coli</i> [†]	29 (1.5)	29	na	15 (1.2)	16 (0.5)	na	18 (0.5)	18 (2.1)	na	21	20 (1.5)	na	21 (0.5)	22 (0.5)	na
	<i>P. aeruginosa</i> [†]	25 (1.5)	25	25 (0.9)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	<i>E. faecalis</i> [†]	ns	ns	ns	ns	ns	ns	12	13 (0.5)	12 (0.5)	33	33	33 (1.3)	8	8	9 (0.5)
	<i>E. coli</i> [*]	37	28 (0.6)	na	14	12 (1.5)	na	19	18 (2.2)	na	25	25 (0.5)	na	20	20 (0.5)	na
Thymol	<i>E. coli</i> [†]	29 (1.5)	na	33	15 (1.2)	na	14	18 (0.5)	na	19	21	na	21	21 (0.5)	na	20
	<i>P. putida</i> [*]	27	na	19.5 (0.5)	30	na	27 (0.5)	ns	na	ns	ns	na	ns	14	na	12 (2.1)
	<i>A. baumannii</i> [*]	19	na	33 (0.5)	19	na	22	ns	na	ns	ns	na	ns	15	na	16 (0.5)
Triclosan	<i>S. aureus</i> [†]	22	21 (0.5)	na	17 (1.5)	21 (0.5)	na	45 (0.5)	51 (2.5)	na	47 (0.5)	44 (0.5)	na	26 (0.5)	34	na
	<i>E. coli</i> [†]	29 (1.5)	41 (1.5)	na	15 (1.2)	13 (0.5)	na	18 (0.5)	18 (0.5)	na	21	28 (0.5)	na	21 (0.6)	20 (1.5)	na
	<i>C. sakazakii</i> [*]	28	32 (0.6)	na	17	20 (0.5)	na	11	12	na	25	21 (0.5)	na	17	17 (0.5)	na
	<i>E. coli</i> [*]	37	35	na	14	15 (1.3)	na	19	20	na	25	24 (1.2)	na	20	23 (2.1)	na

Data show growth inhibition zones (mm) representative of antibiotic susceptibility before (P0) and after 14 passages (P14) in the presence of microbicide/formulation. Antibiotic zones of inhibition were determined before antimicrobial exposure (unexposed; UE) and after antimicrobial exposure to both unformulated (UF) (i.e. simple aqueous solution) and formulated (F) (i.e. with surfactants and sequestrants) microbicides. †, non-drain isolates; *, drain isolates. Statistically significant changes are bold text ($P < 0.05$). Bacteria that did not undergo a ≥ 4 -fold change in MBC were not assessed for changes in antibiotic susceptibility. Where data varied between biological replicates, standard deviations have been given in parentheses (n=6). Combinations of bacterium and antibiotic for which BSAC breakpoints are available are indicated in blue text. According to these, no susceptible bacterium became antibiotic resistant following microbicide adaptation.

Table 2. Correlation across strains in the responses to different antibiotics in the linear mixed effects model.

	AMP	CEP	CIP	KAN	TET
AMP	1	0.95	-0.28	-0.08	0.54
CEP	0.95	1	-0.09	0.03	0.61
CIP	-0.28	-0.09	1	0.54	0.17
KAN	-0.08	0.03	0.54	1	0.73
TET	0.55	0.61	0.17	0.73	1
Key:					

A value of 1 indicates that all organisms respond in a perfectly correlated way to the two antibiotics indicated (either more or less sensitive to both), a value of -1 would indicate a perfect negative correlation with organisms that are more sensitive to one antibiotic. Amp, ampicillin; cep, cephalothin; cip, ciprofloxacin; kan, kanamycin; tet, tetracycline.

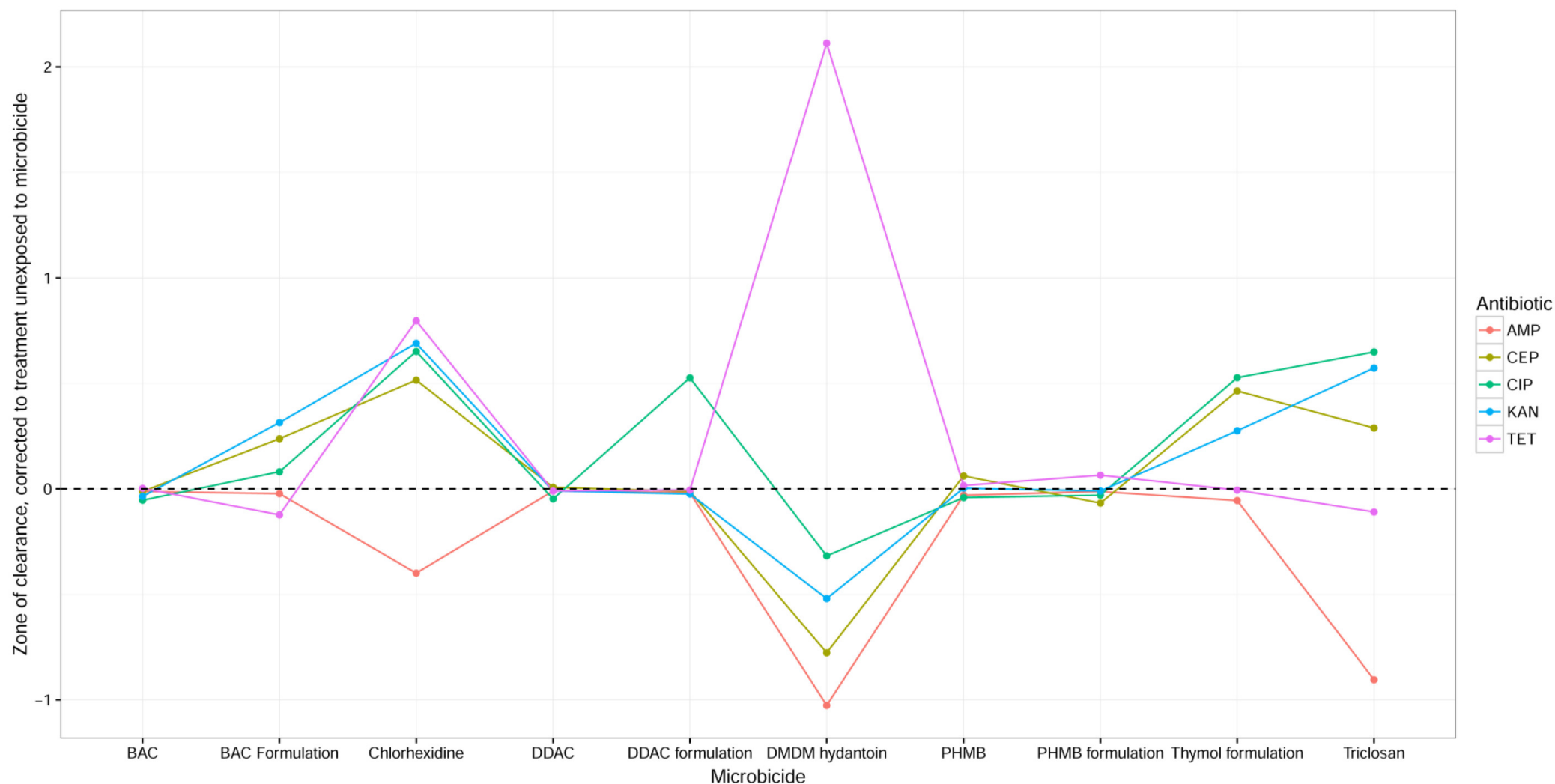


Fig. 1. Antibiotic susceptibility of strains adapted to different microbicides. The values plotted are the difference in the average zone of clearance across strains before and after adaptation to the given microbicide as estimated by the linear mixed effects model (arbitrary scale, see methods). i.e. values above zero indicate antibiotic cross-susceptibility arising from adaptation to microbicide and values below zero indicate cross-resistance. Points are connected for ease of comparison only. See footnote to Tables 1 and 2.

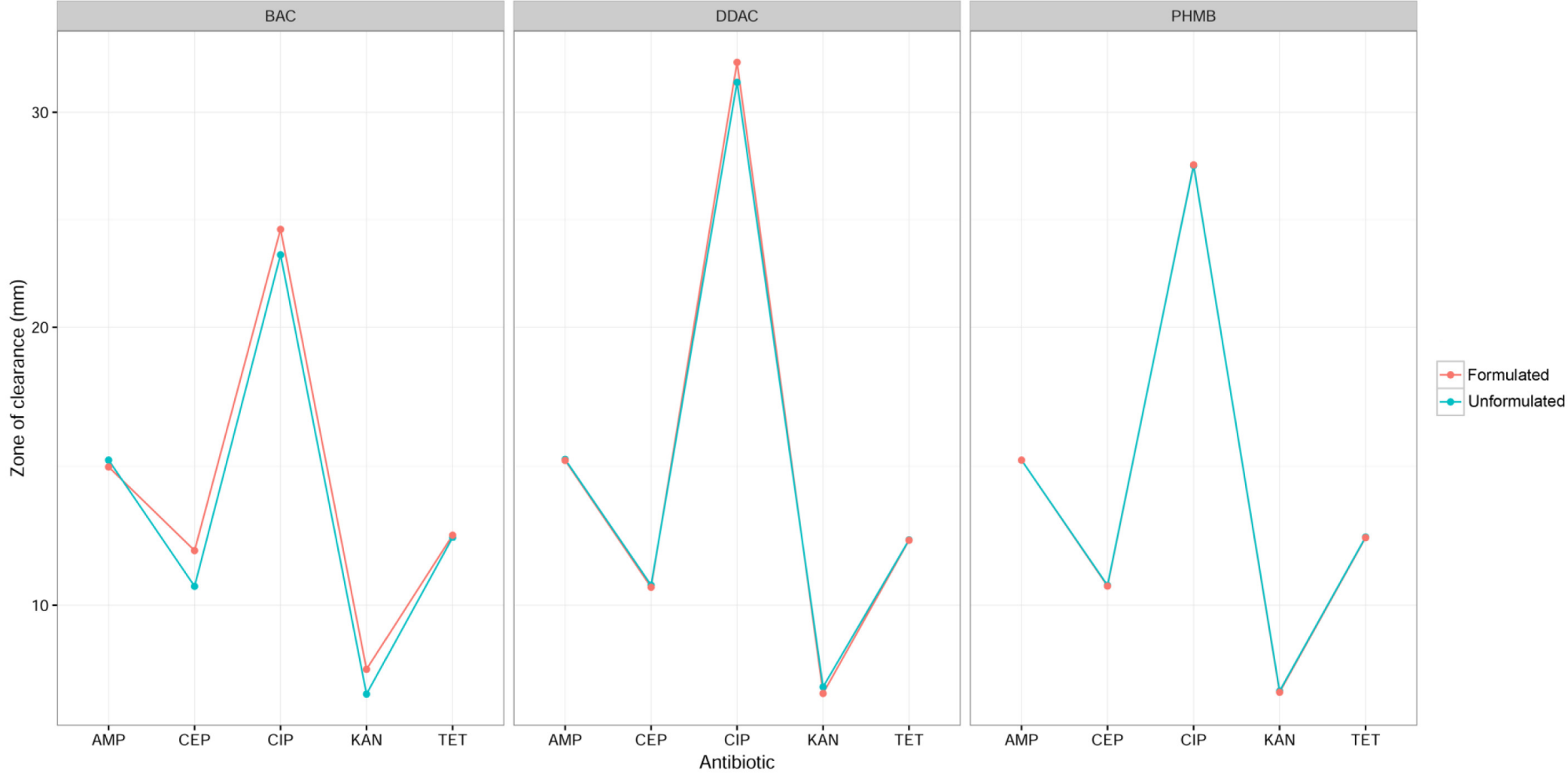


Fig. 2. Antibiotic susceptibility of strains exposed to different microbicides in formulation with surfactants and sequestrants) and simple aqueous solution (unformulated). A significant difference is only apparent for BAC. The values plotted are the average zone of clearance, in mm, as estimated in the linear mixed effects model (note the transformed scale as used by the model, see methods). See footnote to Table 1.